Diversity of *Ochrobactrum* species in food animals, antibiotic resistance phenotypes and polymorphisms in the bla_{OCH} gene

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Abstract

Twenty-six lactose non-fermenting, oxidase, urease and citrate positive Gram negative rods, isolated from broiler chickens, pigs and cattle at slaughter, were subjected to MALDI-TOF mass spectrometry and 16S rDNA sequencing for identification. Susceptibility to 14 antimicrobials was determined by the disc diffusion method. *Ochrobactrum* isolates resistant to third generation cephalosporins were PCR-screened for the presence of the *O. anthropi ampC* gene (bla_{OCH}). A 547 bp internal segment of bla_{OCH} in the *Ochrobactrum* species was amplified with a newly designed primer set and a phylogenetic reconstruction based on the complete amino acid sequence of bla_{OCH} obtained from 9 *Ochrobactrum* strains in our collection and 20 *O. anthropi* available in the GenBank was undertaken. All the *Ochrobactrum* isolates were resistant to the expanded-spectrum beta-lactams and streptomycin. None of the isolates was resistant to imipenem while 41.7% to 50.0% of them were resistant to fluoroquinolones. The bla_{OCH} gene was detected in 16 (66.7%) and 20 (83.3%) of the 24 *Ochrobactrum* isolates using primers designed for *O. anthropi* and the newly designed primer set, respectively. Six AmpC variants grouped into two divergent clusters were identified. This is the first report of the complete nucleotide sequence of the non-*Ochrobactrum anthropi* species bla_{OCH} gene.

Key words: Non-fermenters, *Ochrobactrum* species, ampC, bla_{OCH}, polymorphism, food animals.

Introduction

Ochrobactrum species are non-fermenting, oxidase, urease and citrate-positive Gram-negative rods belonging to the family Brucellaceae (Lebuhn *et al.* 2000; Teyssier *et al*. 2005; Hong *et al*. 2016). They are adapted to various ecological niches (Teyssier *et al*. 2005) and, as such, have been isolated from the soil and water (Lebuhn *et al*. 2000), plants (Tripathi *et al*. 2006), animals (Kampfer *et al*. 2003; ElAdawy *et al*., 2012; Kampfer *et al*. 2011), humans (Velasco *et al*. 1998; Dharne *et al*. 2008; Khan 2014; Hong *et al*. 2016) and wastewater (Adelowo and Fagade 2012; Mustapha *et al*. 2016). *Ochrobactrum* species, particulalry *O.*

anthropi and *O. intermedium*, are increasingly being reported as emerging pathogens capable of infecting both immunocompetent and immunocompromised individuals (Daxboeck *et al*. 2002; Apisarnthanarak *et al*. 2005; Dharne *et al*. 2008; Thoma *et al*. 2009; Wi and Peck 2010; Shrishrimal 2011; Khan *et al*. 2014). Members of this genus exhibit resistance to β-lactam antibacterial agents (Nadjar *et al*. 2001; Soloaga *et al*. 2009; Thoma *et al*. 2009). As pointed out by Cieslak *et al*. (1996), patients infected by *Ochrobactrum* species frequently do not respond to standard empiric antimicrobial treatment due to production of an inducible and chromosomally enconded AmpC-type β-lactamase (Higgins *et al*. 2001; Nadjar *et al*. 2001). This enzyme has been well characterized in *O. anthropi*, but there is a lack of data about its presence and genetic characteristics in other *Ochrobactrum* species. All the DNA sequences regarding the bla_{ampC} gene of *Ochrobactrum* (named bla_{OCH}) that were available in the GenBank database prior to this study were referred to *O. anthropi*.

Unlike in humans, there is limited data on *Ochrobactrum* species from food animals. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been found to be a cost-effective and time-saving alternative method of bacterial identification when compared with conventional 16S rRNA gene sequencing (Bizzini *et al*. 2011; Quirino *et al*. 2014). Both methods have been used in typing of *O. anthropi*. In this study, the identification and antimicrobial susceptibility profiles of *Ochrobactrum* species isolated from food animals at slaughter is reported. Additionally, all isolates showing resistance to extended-spectrum β-lactams were screened for the presence of the *O. anthropi* AmpC β-lactamase-encoding gene (bla_{OCH}) and the complete nucleotide sequence of the different gene variants detected among the studied *Ochrobactrum* species has been provided.

Materials and Methods

Bacterial strains and species identification

Twenty-six non-fermenting, oxidase, urease and citrate positive Gram negative rods, isolated from faecal samples collected from 200 broiler chickens, 100 pigs and 100 cattle presented for slaughter at the Ikpa market/slaughter house, Nsukka, were used in the study. Isolates were sub-cultured on MacConkey agar and incubated at 37°C overnight. Colonies on

Brain Heart Infusion Agar were processed for species identification using the MALDI-TOF MS (Bizzini *et al*. 2011; Quirino *et al*. 2014) and 16S rDNA sequencing. Bacterial genomic DNA was extracted by the InstaGene Matrix kit (BioRad, Hercules, California, USA) and the PCR assay was performed using previously described primers (uni16-F: AGAGTTTGATYMTGGCTCAG; uni16-R: GGYTACCTTGTTACGACTT) (Juretschko *et al.* 1998; Loy *et al.* 2002).

Antibacterial susceptibility testing

Antibacterial susceptibility profiles of the 24 *Ochrobactrum* isolates were determined by the disc diffusion method using 14 antibacterial agents: ciprofloxacin (5µg), ofloxacin (10µg), enrofloxacin (5µg), ampicillin (10 µg), ceftriaxone (30µg), cefoxitin (30µg), ceftazidime (30µg), cefotaxime (30µg), amoxicillin/clavulanic acid (30µg), imipenem (10µg), sulphamethoxazole/trimethoprim (25µg), gentamicin (30µg), streptomycin (10µg), and tetracycline (30µg). Results of the antibacterial susceptibility testing were interpreted according to the Clinical and Laboratory Standards Institute (CLSI 2013) guidelines for veterinary pathogens.

Isolates resistant to extended-spectrum beta-lactam antibacterial agents were screened for extended-spectrum beta-lactamase (ESBL) production using the combination disc method (cefpodoxime/clavulanic acid [10:1 ug] and cefpodoxime alone [10 ug]) on Mueller-Hinton agar. Each test isolate that produced an inhibition zone diameter difference of 5 mm or more between the combination disc and cefpodoxime alone will be considered an ESBL producer.

Detection and characterization of the ampC gene

All the 24 *Ochrobactrum* species isolates exhibiting resistance to third generation cephalosporins were PCR-screened for the presence of the *O. anthropi ampC* gene (*bla*$_{OCH}$) using two different pairs of primers previously described by Nadjar *et al.* (2001) and Higgins *et al.* (2001). After sequencing the obtained amplicons, resulting nucleotide sequences were compared with those available in the GenBank database.

A multiple alignment comparison of the complete AmpC β-lactamase encoding gene was performed with all the different variants obtained from the analyzed *Ochrobactrum* species

strains from this study and also with those available in the GenBank (all belonging to the species *O. anthropi*). Once conserved regions were identified, novel primers were designed (Ochr-IntAmpC-F: CAGCTTCGACAAGATCACCA; Ochr-IntAmpC-R: CTTGAGCGCAGTCGGATAG) in order to allow the amplification of a 547 bp internal segment of the *bla*$_{OCH}$ gene in both *Ochrobactrum anthropi* and non-*anthropi Ochrobactrum* species and this PCR was tested in the 24 *Ochrobactrum* isolates of this study.

MEGA7 software was employed to create a phylogenetic reconstruction based on the complete amino acid sequence of the *bla*$_{OCH}$ gene obtained from 9 *Ochrobactrum* species strains isolated in this study and 20 *O. anthropi* available in the GenBank database.

Nucleotide Sequence Accession Numbers

New variants of the *Ochrobactrum* species *bla*$_{OCH}$ gene were deposited in the EMBL database under accession numbers LT840070 (*O. tritici* strain C8846-N36), LT840071 (*O. intermedium* strain C8853-N48), LT840072 (*O. intermedium* strain C8855-N51), LT840073 (*Ochrobactrum* sp. Strain C8856-N56), LT840074 (*O. tritici* strain C8857-N58) and LT840075 (*O. tritici* strain C8861-N69).

Results and Discussion

Identification and Distribution of Ochrobactrum species

Results of the identification of the 26 non-fermenting, oxidase, urease and citrate positive isolates by 16S rDNA sequencing and by MALDI-TOF MS are presented in Table 1. Twenty-four of the isolates were identified as *Ochrobactrum spp.* by both techniques and the detected species were concordant in 23 of them (*O. intermedium,* 15 isolates; *O. tritici,* 7 isolates; *Ochrobactrum* spp, 1 isolate); a discrepancy in the species description was found in the remaining isolate (*O. intermedium* by 16S rDNA/*O. tritici* by MALDI-TOF MS). Moreover, two of the 26 isolates that were considered as *O. tritici* and *O. intermedium* by MALDI-TOF, were identifed as *Alcaligenes faecalis* and *Pseudochrobactrum assacharolyticum,* respectively, by 16S rDNA sequencing. The 24 *Ochrobactrum* isolates identified by both 16S rDNA sequencing and MALDI-TOF were used for further characterization in this study. *Ochrobactrium* species were

148 isolated from 4 % (8/200), 14 % (14/100) and 2% (2/100) of the broiler chickens, pigs and cattle,
149 respectively. Nine of the *O. intermedium* were recovered from pigs while five and two were from
150 broiler chickens and cattle, respectively. Four of the *O. tritici* were isolated from pigs while three
151 were from broiler chickens. There are few reports on the isolation of *Ochrobactrum* species in
152 food animals. Among the 16 *Ochrobactrum* species so far described, four have previously been
153 reported in animals and birds: *O. anthropi* from turkeys (ElAdawy *et al*. 2012); *O. pectoris* from
154 turkeys (ElAdawy *et al*. 2012), sheep and swine (Kampfer *et al.* 2011) and *O. gallinifaecis* from
155 faecal samples in a chicken farm (Kampfer *et al*. 2003). Thus, in addition to the four previously
156 reported species, food producing animals can also serve as reservoirs of *O. intermedium* and *O.*
157 *tritic*i. This is the first report on isolation of *Ochrobactrum* species from faecal samples of
158 healthy food animals (broiler chicken, cattle and pigs) in Nigeria. *Ochrobactrum* species are
159 emerging pathogens that have been implicated in severe clinical conditions in humans and
160 animals (Kampfer *et al*. 2007; Thoma *et al*. 2009). *Ochrobactrum intermedium* and *O. tritici*
161 reported in this study is among the five *Ochrobactrum* species (others are *O. haematophilum* and
162 *O. pseudogrignonense, O. pseudintermedium*) previously isolated from various human clinical
163 specimens (Holmes *et al*. 1988; Velasco *et al*. 1998; Teyssier *et al*. 2007; Kampfer *et al.* 2007).
164 However, Hong *et al*. (2016) recently reported the first case of human infection with *O. tritici*, a
165 species that was also identified in the present study.
166
167 *Antibacterial susceptibility profile of Ochrobactrum isolates*
168 The 24 *Ochrobactrum* species (identified by both 16S rDNA and MALDI-TOF MS) were
169 all resistant to the beta-lactam/extended-spectrum beta-lactam antibacterial agents -
170 amoxicillin/clavulanic acid, ampicillin, ceftazidime, ceftriaxone, and cefotaxime- and 95.8%
171 were resistant to cefoxitin. Resistance to sulphamethoxazole-trimethoprim, ofloxacin,
172 ciprofloxacin and enrofloxacin was exhibited by 91.7%, 50.0%, 45.8%, and 41.7% of the
173 isolates, respectively. None of the strains was resistant to imipenem. Resistance to the beta-
174 lactam antibacterial agents observed among the isolates in this study is consistent with reports of
175 previous studies (Higgins *et al*. 2001; Thoma *et al.* 2009; ElAdawy *et al*. 2012). All the 24
176 *Ochrobactrum* strains were completely resistant to cefpodoxime alone and cefpodoxime
177 combination discs. This finding supports previous reports that resistance of *Ochrobactrum*

178 species to beta-lactams is due to their intrinsic ability to produce of AmpC β-lactamases (Cieslak
179 *et al*. 1996; Higgins *et al*. 2001; Thoma *et al*. 2009). The *Ochrobactrum* isolates exhibited 12
180 resistance patterns with AMC-AMP-CAZ-CRO-CTX-FOX-CIP-ENR-OFX-CN-S-STX-TE
181 being the dominant pattern (Table 2). They were resistant to 7-13 antibacterial agents tested and
182 this multi-resistance attribute is worrisome because it portends limited therapeutic options for
183 these emerging pathogens capable of causing diseases in both immunocompetent and
184 immunocompromised individuals.
185
186 *Detection, genetic characterization and polymorphic variants of Ochrobactrum spp. bla*$_{OCH}$ *gene*
187 The susceptibility profile of the 24 *Ochrobactrum* spp. strains and the inactivity of inhibitors
188 (clavulanic acid) suggested the involvement of a class C beta-lactamase. We first screened for
189 the presence of *bla*$_{OCH}$ gene by using the primers described by Nadjar *et al.* (2001) and Higgins
190 *et al.* (2001), designed for *O. anthropi*. Sixteen out of the 24 isolates (66.7%) rendered a positive
191 result. After sequencing, only 9 isolates showing a high quality nucleotide sequence were
192 translated to amino acids and included in the AmpC phylogenetic reconstruction (Fig. 1). Two
193 genetically divergent clusters were identified: One grouped all the AmpC enzymes reported for
194 *O. anthropi* in the GenBank database and also two of the isolates from our collection (one
195 *Ochrobactrum intermedium* and one *Ochrobactrum* spp.) and, the other cluster, included most of
196 the remaining non-*anthropi Ochrobactrum* strains of our study, which showed a close genetic
197 relationship in AmpC enzyme.
198 The multiple alignement of the complete AmpC amino acid sequence of all the 15 variants
199 identified in the phylogenetic tree, which includes 6 of our *O. intermedium/tritici/sp* strains and 9
200 *O. antropi* registered in GenBank, revealed a large degree of sequence heterogenity (Fig. 2). The
201 percentage of identity among them was between 89.2% and 99.7%. Remarkably, in comparison
202 with Genbank *O. anthropi* sequences, deletions of up to six amino acids at positions 17-19 and
203 30-32 of the AmpC were identified in many non-*anthropi Ochrobactrum* sequences. However, it
204 is important to note the existance of highly conserved regions, as well as the detection of the
205 consensus sites described for class C β-lactamases (SXSK, YSN and KTG) (Joris *et al.* 1988;
206 Matsumura *et al.* 1998; Nadjar *et al.* 2001; Singh *et al.* 2009) in all the six deduced amino acid
207 sequence variants detected in this study (underlined in Fig. 2). This fact suggests that, in spite of

the divergent evolution of the *Ochrobactrum bla*$_{OCH}$ gene, there has been a strong selective pressure to maintain the sites involved in the enzyme activity.

Finally, based on the conserved regions of the *bla*$_{OCH}$ gene obtained from *Ochrobactrum anthropi* strains available in the Genbank and from 9 non-*anthropi* isolates from our collection, new primers were designed (Ochr-IntAmpC-F: CAGCTTCGACAAGATCACCA; Ochr-IntAmpC-R: CTTGAGCGCAGTCGGATAG) in order to facilitate the detection of *bla*$_{OCH}$, regardless of the species. A PCR using this primer set with the DNA of all the 24 *Ochrobactrum* isolates was conducted and a specific amplificon of 547 bp in 20/24 samples (83.3%) was obtained. This result showed an improvement in the sensitivity of *Ochrobactrum* spp. *bla*$_{OCH}$ gene detection. The negative PCR result exhibited by 4 *O. intermedium* isolates, could be explained by the presence of gene polymorphisms, or by the involvement of a different antimicrobial resistance mechanism.

Overall, this study has shown that multi-resistant *Ochrobactrum* species harbouring ampC enzymes were detected in food animals (broiler chickens, cattle and pigs) slaughtered in Nsukka Southeast, Nigeria. *Ochrobactrum intermedium* was found to be the dominant species colonizing these animals in the study area. Polymorphism, consisting of deletions of up to six amino acids at positions 17-19 and 30-32 of the AmpC was identified in many of the studied isolates. This is the first report of the complete nucleotide sequence of *bla*$_{OCH}$ gene variants detected in non-*Ochrobactrum anthropi* species.

Food animals in the study area, especially pigs and broiler chickens, are potential reservoirs and disseminators of multi-resistant *Ochrobactrum* species and genes encoding AmpC production. Isolation of these multi-resistant bacteria from food animals has public health significance.

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Figure Legends

Fig. 1. A neighbor-joining phylogenetic tree inferred from the AmpC amino acid sequence of *O. anthropi* strains obtained from the GenBank database and 9Ochrobactrum species isolated in this study (marked with a circle).

Fig. 2. Alignement of *Ochrobactrum* spp. AmpC amino acid sequence variants. Differences are shaded in grey. The consensus sites of class C β-lactamases (SXSK, YSN, KTG) are underlined.

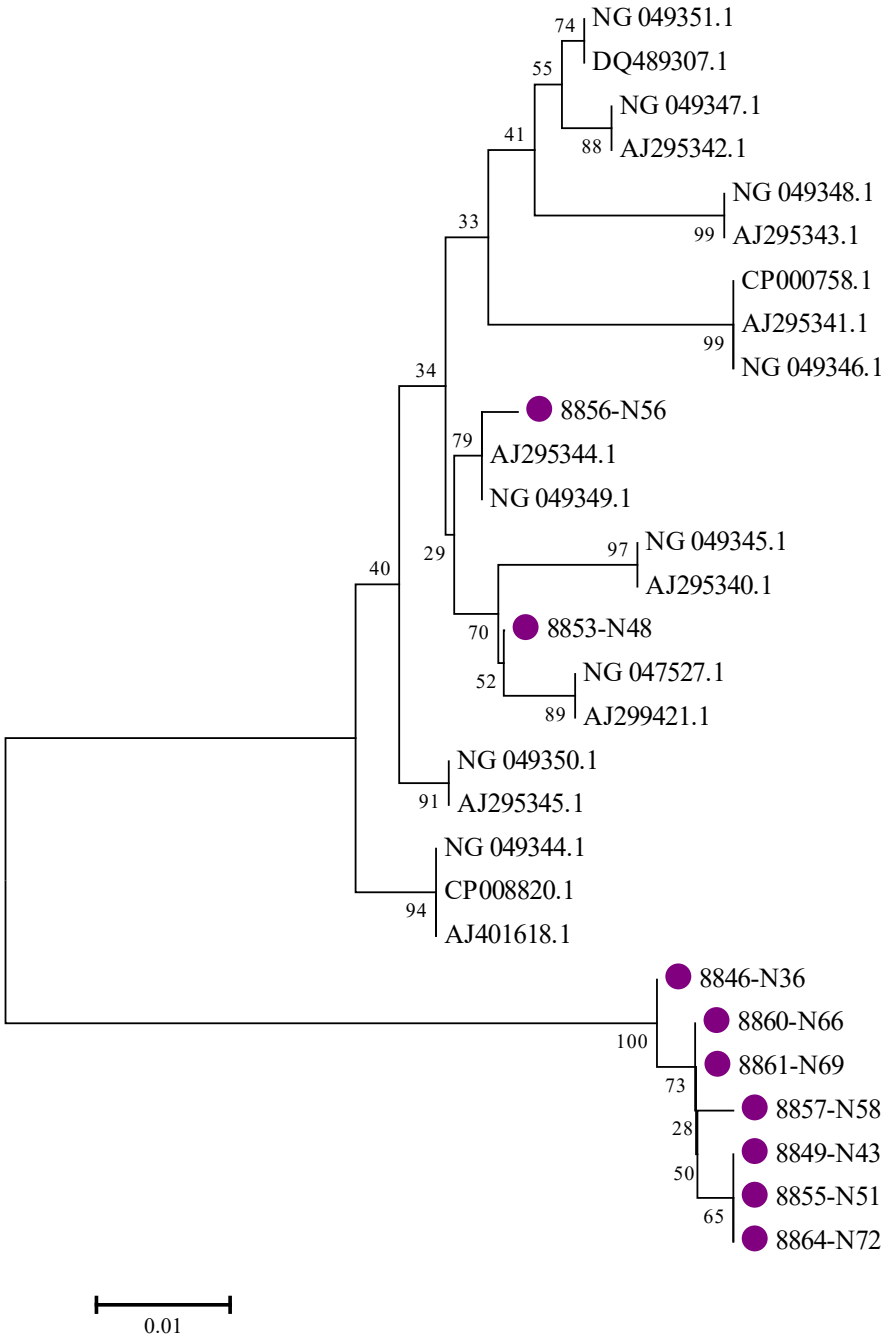
Table 1. Comparative identification of non-fermenting, oxidase, urease and citrate positive rods isolated from broiler chickens, pigs and cattle at slaughter.

Strain Number	Source	16S rDNA sequencing analysis	MALDI-TOF MS
C8839-N24	Broiler chicken	*Ochrobactrum tritici*	*Ochrobactrum tritici*
C8840-N27	Pig	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8841-N28	Pig	*Alcaligenes faecalis*	*Ochrobactrum tritici*
C8842-N29	Pig	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8843-N31	Pig	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8844-N32	Pig	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8845-N35	Pig	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8846-N36	Broiler chicken	*Ochrobactrum tritici*	*Ochrobactrum tritici*
C8847-N37	Pig	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8848-N40	Cattle	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8849-N43	Broiler chicken	*Ochrobactrum tritici*	*Ochrobactrum tritici*
C8850-N45	Broiler chicken	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8851-N46	Pig	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8852-N47	Cattle	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8853-N48	Pig	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8854-N49	Broiler chicken	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8855-N51	Pig	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8856-N56	Pig	*Ochrobactrum* spp.	*Ochrobactrum* spp.
C8857-N58	Broiler chicken	*Ochrobactrum intermedium*	*Ochrobactrum tritici*
C8859-N63	Broiler chicken	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*
C8860-N66	Pig	*Ochrobactrum tritici*	*Ochrobactrum tritici*
C8861-N69	Pig	*Ochrobactrum tritici*	*Ochrobactrum tritici*
C8862-N70	Pig	*Ochrobactrum tritici*	*Ochrobactrum tritici*
C8863-N71	Broiler chicken	*Pseudochrobactrum assacharolyticum*	*Ochrobactrum intermedium*
C8864-N72	Pig	*Ochrobactrum tritici*	*Ochrobactrum tritici*
C8865-N84	Broiler chicken	*Ochrobactrum intermedium*	*Ochrobactrum intermedium*

Table 2. Resistance patterns exhibited by the 24 *Ochrobactrum* isolates from food animals.

S/N	Resistance Pattern[a]	Number of isolates	Percentage
1	AMC-AMP-CAZ-CRO-CTX- S-SXT-TE	1	4.2
2	AMC-AMP-CAZ-CRO-CTX-FOX-CIP-CN-S-SXT-TE	1	4.2
3	AMC-AMP-CAZ-CRO-CTX-FOX-CIP-ENR-OFX-CN-S-SXT-TE	8	33.3
4	AMC-AMP-CAZ-CRO-CTX-FOX-CIP-ENR-OFX-S-SXT-TE	1	4.2
5	AMC-AMP-CAZ-CRO-CTX-FOX-CIP-ENR-S-SXT-TE	1	4.2
6	AMC-AMP-CAZ-CRO-CTX-FOX-CN-S-SXT-TE	3	12.5
7	AMC-AMP-CAZ-CRO-CTX-FOX-CN-S-TE	1	4.2
8	AMC-AMP-CAZ-CRO-CTX-FOX-OFX-CN-S-SXT-TE	2	8.3
9	AMC-AMP-CAZ-CRO-CTX-FOX-OFX-S-SXT	1	4.2
10	AMC-AMP-CAZ-CRO-CTX-FOX-S	1	4.2
11	AMC-AMP-CAZ-CRO-CTX-FOX-S-SXT	3	12.5
12	AMC-AMP-CAZ-CRO-CTX-FOX-S-SXT-TE	1	4.2

[a] AMC: Amoxicillin/Clavulanic acid; AMP: Ampicillin; CAZ: Ceftazidime; CRO: Cetriaxone; CTX: Cefotaxime; IMP: Imipinem; FOX: Cefoxitin; CIP: Ciprofloxacin; ENR: Enrofloxacin; OFX: Ofloxacin; CN: Gentamicin; S: Streptomycin; SXT: Sulphamethoxazole-trimethoprim; TE: Tetracycline.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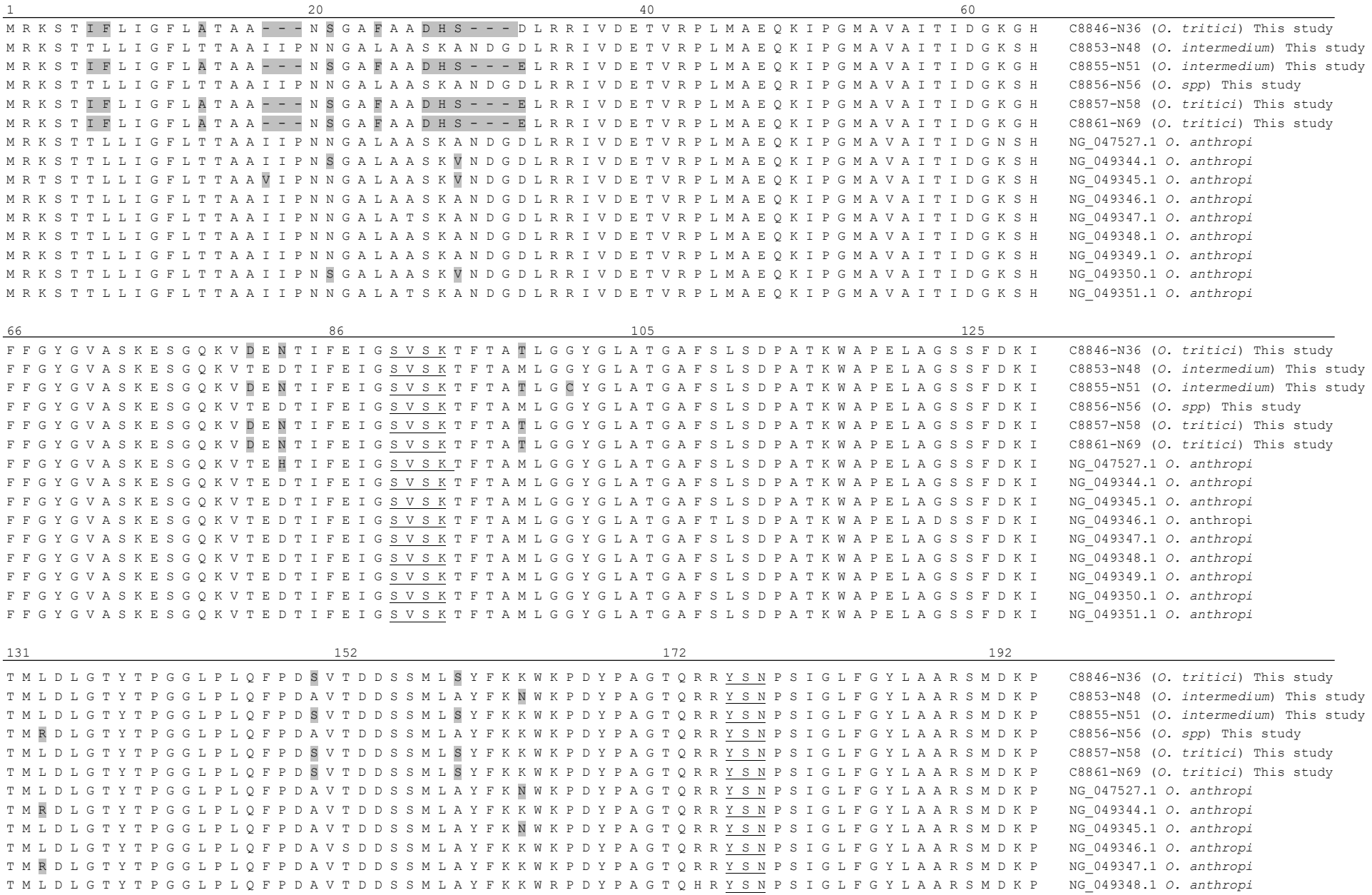
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196                                 218                                 238                                 258
F D V L M E Q K L L P A F G L T N T F V N V P E S R M K N Y A Y G Y S K T D K P I R V S G G A L D A Q A Y G I K T T A P D L I R F    C8846-N36 (O. tritici) This study
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261                                 284                                 304                                 324
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V E L N I D S S S L E P D F Q K A V A A T H T G Y Y H V G A N N Q G L G W E F Y N Y P T A L K T L L A G N S S D M A L K S H K I E    NG_049349.1 O. anthropi
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V E L N I D S S S L E P D F Q K A V A A T H T G Y Y H V G A N N Q G L G W E F Y N Y P T A L K T L L A G N S S D M A L K S H K I E    NG_049351.1 O. anthropi

326                                 350                                 370                                 390
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